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(54) Title: METHOD FOR DETERMINING ANTIGENS

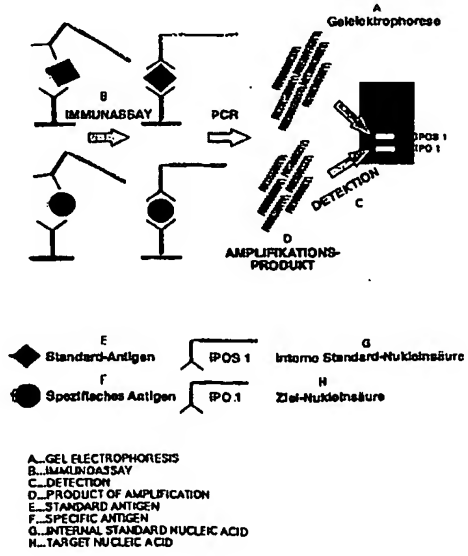
(54) Bezeichnung: VERFAHREN ZUR BESTIMMUNG VON ANTIGENEN

(57) Abstract

The invention relates to a method for determining antigens in a sample by amplifying a nucleic acid, said nucleic acid being bonded to a ligand which is specific to the antigen. The determination is carried out in the presence of at least one internal standard.

(57) Zusammenfassung

Beschrieben wird ein Verfahren zur Bestimmung von Antigenen in einer Probe durch Amplifizieren einer Nukleinsäure, die an einen für die Antigene spezifischen Liganden gebunden ist, wobei die Bestimmung in Anwesenheit mindestens eines internen Standards durchgeführt wird.



Method for the detection of antigens

ABSTRACT:

A method for the detection of antigens in a sample by amplifying a nucleic acid bound to a ligand specific for said antigens is described, with the detection taking place in the presence of at least one internal standard.

Fig. 1

The invention relates to a method for the detection of antigens in a sample by amplifying a nucleic acid which is bound to the antigen to be detected.

The detection of specific antigens is particularly difficult in low concentration ranges. However, the very antigens to be analyzed often have to be detected in low concentration ranges or amounts in order to obtain important information, e.g. on clinical pictures, biological contaminations, physiological values of selected proteins etc..

Antigens are usually detected by means of antibody tests, e.g. ELISA. In such tests, the antigen of interest is detected by being bound to an antibody specific for said antigen. The amount of bound antibody/antigen is then usually determined by an established detection method, such as e.g. radioactivity, fluorescence, staining reaction, etc., preferably using secondary antibodies.

However, even the most sensitive ELISA tests, e.g. those using radioactivity detection methods, have a limit of detection which, in some cases, is too high by some powers of ten to yield useful results. Furthermore, there are proteins or pathogens which can not be detected at all or not to a satisfying extent by means

of conventional antibody/antigen detection methods, due to the specific nature of the antigen.

One example of such a pathogen is the antigen responsible for scrapie. Scrapie was first discovered in sheep some 250 years ago. In recent years, scrapie-related diseases have also been described in other animals and also in humans as transmissible spongiform encephalopathies (TSE). These diseases are assumed to be caused by prions. Prion-related diseases can not be diagnosed satisfyingly by means of conventional antigen/antibody tests.

Prion proteins are either non-infectious, called PrP^{c}, or infectious, called PrP^{Sc}. The non-infectious prion protein PrP^{c} is a ubiquitous cellular protein which turns into the infectious prion protein PrP^{Sc} due to an alteration in its conformation. In order to distinguish the normal prion protein from the infectious prion protein, usually proteinase K digestion is carried out, followed by Western blotting; the infectious prion protein PrP^{Sc}, however, has shown to be partly resistant to proteinase K digestion. Moreover, Korth et al. (Nature 390 (1998), 74-77) describe a method for the preparation of antibodies which specifically recognize either the "normal", i.e. non-infectious prion protein, or specifically recognize the infectious prion

protein, respectively.

Prions cause mostly progressive diseases of the central nervous system (spongiform encephalopathies), e.g. scrapie in sheep, bovine TSE, kuru, and Creutzfeld-Jakob disease (CJD), Gerstmann-Sträussler syndrome (GSS) and fatal familiar insomnia (FFI). Prions differ from viruses and bacteria in particular physicochemical and biological properties: they are highly resistant to inactivation by means of formaldehyde, nucleases, heat, UV and ionizing irradiation, and they are not detectable as virions or cells in the electron microscope. They are insensitive to interferons, do not show interference with viruses, and no nucleic acid has been detected so far.

Recently, in Great Britain a variety of CJD involving new neuropathological features (nvCJD) has appeared more frequently. It has been shown that this nvCJD was caused by the tranmission of BSE from cattle to humans consuming contaminated beef offal. Therefore, it has frequently been demanded that biological materials used for the preparation of therapeutic compositions, such as e.g. body fluids, blood, plasma, plasma fractions and serum, tissue and cell cultures, be assayed for prions.

Hence, assaying of biological materials and subsequently discarding contaminated material is gaining more and more importance as prions have proven to be highly resistant to conventional inactivation methods, such as e.g. heat or UV treatment.

Conventional prion tests, such as e.g. Western blotting using specific antibodies, are not sufficiently sensitive to determine prion protein effectively. WO 97 37 227 describes an immuno histochemical test for the detection of prions in tissues. By means of this method, the altered prion protein (PrP^{Sc}) may probably be detected in histological sections from infected tissue parts. This method, however, is not very reliable, and it is particularly unsuitable for examining blood and blood products.

Polymerase chain reaction (PCR) is a method for the in vitro amplification of specific single or double stranded DNA fragments. Repeated heat denaturation of DNA double strands, hybridisation (annealing) of the primer and extension by means of DNA polymerase result in an enormous enrichment of a double stranded DNA fragment. After 20 cycles, about 1 million copies will be obtained from one DNA molecule due to an exponential chain reaction (cf. EP 0 200 362 and EP 0 201 184). However, only nucleic acids can be detected by means of

this method, which is unsuitable for proteins.

An improved method for PCR is described in EP 0 714 988. In this method, a standard is added to the sample to be assayed to allow quantitation of the DNA determined by means of PCR.

Sano et al. (Science 258 (1992), 120-122) describe a combination of ELISA and PCR as "immuno PCR". In this method, a connecting molecule (linker) having bispecific binding affinity for DNA and antibody, e.g. a chimeric molecule of streptavidin for biotinylated DNA and protein A for immunoglobulin G, is used to bind a certain DNA molecule (marker) unspecifically to an antigen-antibody complex, thus forming an antigen-antibody-linker-DNA complex. The bound DNA marker can now be amplified by means of appropriate primers in a PCR. The detection of the PCR product will prove the presence of the antigens.

The labelled DNA-antibody complexes are put together in situ in the reaction mixture, which may result in different stochiometries in the composition and binding of the DNA. Additional steps for the addition of biotinylated reagents and binding proteins as well as numerous washing steps are required to remove excessive reagents. These numerous method steps, however, make this

method too complicated and time-consuming to be employed in routine analyses. Moreover, this method is unsuitable for complex mixtures of various antigens.

Hendrickson et al. (Nucl. Acids Res. 23(3) (1995), 522-529) describe an improved immunoassay for the simultaneous detection of various analyts. In this method, the antibodies and the DNA, a single strand oligonucleotide in this case, are activated separately and coupled directly in a spontaneous reaction. Hendrickson et al. describe the simultaneous detection of three different analyts by means of immuno-PCR. The DNA-labelled antibodies bound to the corresponding antigen are amplified and determined by means of PCR.

However, like PCRs in general, these immuno PCR methods always involve the risk of falsely negative results, i.e. a specific antigen cannot be detected by this detection reaction although said specific antigen is actually present in the sample. The consequences may be fatal, particularly when detecting pathogens in samples to be processed into medicaments to be administered to humans: due to the falsely negative results, some samples might be selected for processing although they actually contain pathogens.

In order to eliminate this risk of using possibly in-

fectious starting materials in the preparation of pharmaceutical preparations, efforts are being made to rule out falsely negative results.

It is therefore the object of the present invention to provide a reliable and sensitive method for the detection of antigens which primarily eliminates falsely negative results a priori.

Said object of the present invention is solved by a method for the detection of antigens by amplifying a nucleic acid (target nucleic acid), which is bound to a ligand specific for said antigens; the method is characterized in that the detection is carried out in the presence of at least one internal standard.

By means of the method according to the invention, antigen detection using immunoamplification reactions can be monitored and controlled elegantly. Primarily, however, the method according to the present invention reliably eliminates falsely negative results. Moreover, the use of internal standards also allows obtaining a value for quantitative comparison for antigen detection in the sample, particularly if more than one standard is used. In routine practice, however, it will usually not be necessary to use more than one standard, particularly if a standard is used only in order to

eliminate falsely negative results.

According to the invention, preferably a nucleic acid bound to a ligand is used as a standard (standard nucleic acid). Particularly preferably standard nucleic acids are used which differ from the nucleic acid used for the detection of the (target) antigen (target nucleic acid) in at least one detectable feature.

According to the invention, subsequently both the standard and the target nucleic acids are amplified, and the amplified nucleic acids are assayed.

Examples for (gene) amplification methods are, apart from polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), ligase chain reaction (LCR) and nucleic acid sequence based amplification (NASBA).

In the "Taqman assay" the polymerase chain reaction is combined with an hybridization of the target sequence using a labelled probe in order to allow better characterization of the samples (Holland et al., Proc. Natl. Acad. Sci., 88 (1991), 7276-7280).

The standard nucleic acid may preferably have a length of at least 70 nucleic acids. Preferably a length of from >100 to several hundred nucleic acids is chosen.

The target nucleic acid is bound to the ligand specific for the antigen to be detected. Preferably antibodies specific for the antigen to be detected are used as ligands. Affinity ligands may be used as well, such as e.g. peptides binding specifically to the antigen. The standard nucleic acid usually differs from the target nucleic acid in at least one detectable feature, although preferably it is amplified by the same means, such as primers. Standard nucleic acids being different in size from the nucleic acid to be detected or having a unique restriction cleavage site have proven preferable, because if they are used the result can be analyzed by simple gel electrophoresis. Preferred standards differ from the nucleic acid to be detected by 1 to 20% of their length or in at least 3 and not more than 50 nucleotides; a standard nucleic acid longer than the target nucleic acid is referred to as "plus" ("+") standard, and a standard nucleic acid shorter than the target nucleic acid is referred to as "minus" ("-") standard. The exact sequence of the standard nucleic acid should be known, of course.

Both the nucleic acid for the internal standard and the nucleic acid which is bound to the ligand specific for the antigen to be detected may be employed either as a double strand or as a single strand nucleic acid. The

preferred choice is a single strand DNA having a terminal amino group for chemical crosslinking with the specific ligand. Nucleic acids having a length of 70 to 120 base pairs are preferably used.

The internal standard may also be provided as a combination of the standard nucleic acid and the ligand specific for a standard antigen. The internal standard may either be bound directly to the specific ligand, or the linkage between the specific ligand and the DNA may be formed via a connecting molecule (linker) which has, on the one side, a binding site for the DNA, e.g. streptavidin, and, on the other side, a specific ligand directed against an antigen (standard antigen). In a preferred embodiment the DNA is bound directly to the ligand via a chemical bond, e.g. an affinity bond or a covalent bond.

Peptides or monospecific antibodies, monoclonal or polyclonal, but also chimeric, recombinant or humanized antibodies, respectively, may be used as ligands for the internal standard and/or for the antigen to be detected. The ligands, which are bound to the standard nucleic acid, may also be directed specifically against the specific antigen in order to act specifically for the antigen to be detected in competition with the target ligand. Polyspecific antibodies recognizing the

standard antigen may also be used. Ligands specific for the antigen to be detected are preferably antibodies appropriate for the detection of modified proteins and prion proteins. Similarly, ligands specific for the antigens to be detected may also be chosen for other purposes, e.g. from the group of viral, bacterial or human proteins. For the detection of prions, preferably antibodies specific for a prion protein are used.

Primers used in the amplification may preferably contain markers which further extend the limit of detection of the amplified nucleic acids, e.g. fluorescent or radioactive groups or chemical groups detectable by means of affine proteins and subsequent detection reactions (e.g. biotin-avidin, DIG labelling etc.); primers having fluorescent groups are particularly preferred.

The amplified nucleic acids may be detected in a variety of ways, though mostly a step is required wherein the amplified standard nucleic acid is separated from the amplified target nucleic acid, and the separated nucleic acids are determined individually. Preferably this separation step is a gel electrophoresis step or a chromatographic process. Determination of the ratio of the amount of target nucleic acid and the known amount of standard nucleic acid allows, in addition, a quantitation of the antigen.

Among the antigens detectable by the method according to the invention are antigens from biological mammalian samples, such as body fluids, e.g. plasma, blood, blood cells and serum, tissue, but also antigens from eucaryotic cell cultures. According to the invention, antigens of pathogens or marker antigens for pathogens are preferably detected. Among these antigens are viral antigens, bacterial antigens and particularly antigens for which a routine detection method has not been available, such as e.g. modified proteins (e.g. having an altered conformation), such as prions involved in infectious diseases of the central nervous system, such as CJD and BSE, or other TSE agents. Such modified proteins may be obtained by means of induced conformation alterations. Said alterations in the conformation of proteins, such as optionally recombinant PrPc, may be caused by previous incubation with infectious molecules (e.g. PrPSc), which will then be detectable by radioactive markers, e.g.. Such an alteration in the conformation of modified prion molecules has been described by Kocisko et al. (Nature 370 (1994), 471-474).

In a preferred embodiment, one or more antigens of human pathogens, such as HIV, hepatitis viruses including HAV, HBV, HCV, HGV and parvoviruses, are detected. The

antigens to be detected may be detected in a purified preparation containing protein, e.g. a plasma fraction, a preparation of a purified plasma protein, and a biological medicament, respectively. They may also be reliably detected in plasma pools or other complex mixtures and in the above-mentioned biological samples. Quality management, as described in WO 96/35437, may also be carried out by appropriately adapting the method of the present invention.

The appropriate internal standard may be added before preparing the samples, during all intermediate steps or directly before the amplification reaction. In a preferred embodiment, the standard is added from the beginning as an unbound nucleic acid or as a bound nucleic acid after immune complex formation, respectively. Moreover, two (or more) different nucleic acids may also be added as an internal standard. Preferably the internal standard is added at a concentration slightly above the limit of detection. The embodiments described in EP-0 714 988-A can be readily adapted for the present invention.

The antigen bound (recognized) by the internal standard may be added to the sample to be detected (e.g. the antigen detection mixture), or it may be present in the sample naturally (a priori).

In routine tests, the results obtained by means of the method according to the invention may usually be interpreted as follows:

i) No detection of the internal standard (e.g. no visible bands): Detection did not work, e.g. due to the amplification reaction (e.g. PCR); falsely negative results can thus be ruled out.

ii) Only the internal standard is detectable (e.g. only the standard band is visible): Detection including the amplification reaction (e.g. PCR) did work, the sample is negative.

iii) Standard and sample nucleic acids are detectable (e.g. both bands are visible): Positive sample.

Suitable reaction vessels for the immuno PCR should preferably have good protein binding capacity to immobilize the proteins. On the other hand, the vessels must also be thermostable for the PCR. For a large number of samples microtiter plates are preferred to individual reaction vessels. Regarding protein binding, polystyrene is the most appropriate material, but so far such plates suitable for PCR have not been commercially available. Polycarbonate plates, for instance, may be used as a substitute according to the invention. The use of chemically modified materials for the covalent bonds of proteins or materials already coated with

antibodies may also be considered.

The plates are preferably coated overnight at 4°C, although higher temperatures of up to about 37°C and shorter incubation periods are also possible. As a coating buffer, e.g. PBS (phosphate buffered saline solution) as well as any other common buffer, e.g. polycarbonate buffer, may be used.

Blocking may be effected e.g. by means of PBS/1% BSA (bovine serum albumin). Any other suitable reagent, e.g. milk powder or ready-made blocking reagents, may be used as a substitute.

Incubation with the ligand/DNA complex is preferably carried out for half an hour at room temperature, although other periods of time (preferably 5 min to 2 hours) and other temperatures (preferably 4°C to 37°C) are possible as well.

Optimum reaction conditions for the PCR are described in example 1. Of course, a large number of changes is possible (particularly changes in the number of cycles, incubation periods, temperature profile, use of different polymerases, amplification systems, primer strategies etc.). If, for instance, the protocol used in the

examples should prove to be not optimal, optimum conditions for the individual antigens to be detected can be readily determined by a person skilled in the art.

A further aspect of the present invention is related to the use of the method according to the invention for assaying and quality management of biological preparations.

In principle, the safety, particularly the virus safety, of stable blood products depends on the extent of pathogen contamination, particularly on the virus contamination of the starting material, on the depletion capacity of the method and on the specific virus inactivation steps within the production process. An important criterion for quality management is therefore the exclusion of positive starting materials from being processed into pharmaceutical medicaments.

Thus, thanks to the high sensitivity and the particularly low limit of detection of the method according to the invention, new quality criteria for biological products can be set up which are defined by an extremely low defined or lacking content of contaminating antigens.

A further aspect of the invention is a reagent to be

used according to the method of the invention, which includes:
a) a reactant bound to a ligand specific for the antigen to be detected, and
b) a reactant as an internal standard differing from reactant a) in its way of reaction.

Different ways of reaction may, for instance, be different reaction rates and specifities for different reactants. Nucleic acids or enzymes may be used as reactants, e.g. to obtain a controlled enzyme reaction. In a preferred embodiment, nucleic acids are used as reactants which differ in one detectable feature, e.g. in length.

Particularly if the reagent of the invention is employed in the detection of modified proteins, the target nucleic acid is preferably present bound to an antibody specific for the modified protein.

According to a preferred embodiment of the reagent of the invention, the standard nucleic acid is bound to a ligand, particularly to a ligand already bound to a standard antigen. The standard antigen may be the same as the antigen to be detected (competitive immune reaction).

The standard nucleic acid in the reagent of the invention is preferably bound to a ligand specific for an antigen different from the antigen to be detected (target antigen).

The ligands/DNA complexes may be stored after preparation at
-20°C or 4°C in a suitable buffer, preferably PBS. Thus they will be stable for several months. This preparation corresponds to a commercial form. Optimum concentration for the use of the complexes is about 10 000 to about 1 000 000 copies per well in a microtiter plate. Preferably less than 1 million copies are used in order to avoid unspecific linkages in the wells. Common formulation reagents, such as e.g. albumin as a carrier protein, surfactants, etc., may be used for the preparation of the reagent according to the invention.

Finally, the present invention also relates to the use of the method according to the invention for quantifying antigens, particulary for quantifying pathogens in mammalian samples or human pathogens in biological medicaments.

The invention will be described in greater detail using the following examples and the drawing figure, with the invention not being restricted thereto, of course.

Fig. 1 shows a schematic representation of IMMUNO-PCR with internal standard.

According to Fig. 1, a microtiter plate is coated with an antibody specific for a standard antigen and an antibody specific for the antigen to be detected. By these antibodies the standard antigen and the antigen to be detected are bound. Subsequently, the ligands are bound to the antigens. The ligands consist of the respective specific antibody for the standard antigen, covalently bound to an oligonucleotide (=internal standard nucleic acid, IPOS 1), and an antibody specific for the antigen to be detected, covalently bound to an oligonucleotide of different length (=target nucleic acid, IPO 1). After a polymerase chain reaction (PCR), the resultant amplification products of different lengths are detected by means of gel electrophoresis.

EXAMPLE 1: Addition of a DNA standard to PCR

In contrast to most ELISAs which are very reliable in principle, in PCR it is quite possible that the reaction will be disturbed by inhibiting substances. This may result in a falsely negative PCR. In the present experiment, the PCR part of the immuno PCR was checked for falsely negative reactions according to the inven-

tion by adding a standard nucleic acid differing in length from the nucleic acid of the antibody/nucleic acid conjugate specific for an antigen.

In this example, a cellular (non-infectious) prion protein (PrP^c) is used as the antigen. This prion protein is not present in purified form but in a homogenate obtained from a mouse brain. For this purpose, the brain was homogenized in a Dounce in a buffer (0.1 g/ml) consisting of a 0.32M solution of cane sugar, 0.5% deoxycholate and 0.5% NP-40. After a centrifugation step (15 min at 4500 rpm) the pellet was discarded, then the amount of protein in the supernatant was determined.

After determination of the protein concentration, different amounts of the homogenate were incubated in 2 similar set-ups (2x uncoated, 2x 8 µg, 2x 800 ng) in polycarbonate plates (96 wells, V-shaped, from MJ Research, Watertown, Mass., USA) overnight in a volume of 50 µl at 4°C. After this coating, the respective wells were blocked at room temperature for 1 hour with 50 µl of a PBS/1% BSA solution. This was followed by half an hour of incubation with about 10^4 copies of the prion antibody/DNA complex (target nucleic acid, 6H4-IPO1; cf. table 1) in 50 µl of PBS/0.1%. After washing 6

times with 100 µl of PBS, the PCR solution was added directly to the respective wells. The PCR solution (50 µl) contained 1 U of Polymerase AmpliTaq Gold™ (Perkin Elmer, Norwalk, CT, USA), 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.001% of gelatin, 200 µM of each dNTP and 500 ng of each of the appropriate primers IP1/IP2 (cf. table 1). The samples were overlaid with 50 µl of mineral oil and, at first, incubated for 14 min at 94°C to activate the polymerase. This was followed by 40 cycles of amplification in a PTC0200 Thermocycler (MJ Research) according to the following profile: 30 s at 94°C, 30 s at 55°C, 60 s at 72°C and a final elongation step at 72°C for 60 s. About 100 and about 1000 copies, respectively, of the internal standard nucleic acid IPOS1 (cf. table 2) were added to the sample preparations, which were checked for falsely negative results.

8 µl of the respective sample preparation were applied to a 3.5% low melting agarose gel. The result of the experiment is given is table 2. It showed that in the wells containing no homogenate only the standard IPOS1 is visible, i.e. there is no specific antigen present in the sample. The reaction, however, was certainly not falsely negative. By adding different amounts of IPOS1,

a competition with the target nucleic acid IPO1 becomes apparent, i.e. 1000 copies give a stronger band than 100 copies. Therefore, the standard should be used at concentrations slightly above the limit of detection in order to avoid knock-out competition of the specific signal. In the case of larger amounts of the specific antigen, it may be quite possible that only the IPO1 band is discernible. This is absolutely as intended in the present invention, because its main object is to avoid falsely negative reactions.

Table 1: Sequences of the oligonucleotides and primers used:

IPOS1 5'-AGAACCCACT GCTTACTGGC CTTATCGAAA TTAATACGAC TCACTATAGG GAGACCCAAG CTTGGTACCG AGCTCGGATG TGCCTTCTAG TTGCCAGCC-3'

IPO1 5'-AGAACCCACT GCTTACTGGC CTTATCGAAA TTAATACGAC TTGGTACCG AGCTCGGATG TGCCTTCTAG TTGCCAGCC-3'

IP1 5'-AGAACCCACT GCTTACTGGC-3'

IP2 5'-GGCTGGCAAC TAGAAGGCAC-3'

The wells of a microtiter plate were coated with brain

homogenate, blocked and incubated with 6H4-IPO1. After washing 6 times, amplification was effected after adding internal DNA standard. The negative control of the PCR reaction was negative, the positive controls of the two DNA reagents used (DNA standard, 6H4-IPO1) were positive. The results are shown in table 2.

1000 copies of the standard nucleic acid IPOS1 were added to samples 1-6, and 100 copies of IPOS1 were added to samples 7-12. Coating was effected with either 8 or 0.8 µg of homogenate.

Table 2: Controlled detection of prion antigen in a mouse brain homogenate

			Bands	
Sample	*Homogenate*	*IPOS1*	*IPO1*	*IPOS1*
	in µg	*copies added*		
1	-	1000	-	++
2	-	1000	-	++
3	8	1000	+	++
4	8	1000	+	++
5	0.8	1000	+	++
6	0.8	1000	+	++
7	-	100	-	+
8	-	100	-	+
9	8	100	+	+
10	8	100	+	+
11	0.8	100	+	+
12	0.8	100	+	+

Explanation of the symbols: + = band, ++ = strong band, - = no band.

Addition of marker antigen:

If the entire immuno PCR is to be checked for falsely negative reactions, a standard nucleic acid will not be added to the PCR, but at the beginning of the experiment. A barely but still detectable amount of a marker antigen as an internal standard will be added to the respective wells of a microtiter plate simultaneously with the specific antigen and incubated overnight at 4°C. This is to make sure that any disturbing influences which might cause falsely negative reactions (including e.g. forgetting to coat a well) are under control. Incubation is effected with the specific anti-

body/DNA complex and simultaneously with the corresponding standard antibody/DNA complex, followed by amplification with the same pair of primers.

If the immuno PCR is based on a so-called Sandwich ELISA, the wells of the microtiter plate must be coated correspondingly with an antibody for the antigen to be detected and an antibody for the standard antigen.

It may also happen that the standard antigen exists naturally, such as, for instance, certain proteins (e.g. protein C) when using biological fluids, e.g. plasma.

EXAMPLE 2: Preparation and use of antibody/DNA complexes:

The preparation of an antibody/DNA conjugate by covalently binding an oligonucleotide to a monoclonal anti-prion antibody followed Hendricksson et al. An amino-modified reporter oligonucleotide (IPO1, prepared by Metabion, Martinsried, Germany) and the monoclonal anti-prion antibody 6H4 (Prionix, Basel, Switzerland) were used as starting materials for the preparation of the conjugate.

Step 1: Preparation of acetylthioacetyl derivatized DNA

Acetylthioacetyl derivatized DNA was prepared by a reaction with succinimidyl-S-acetylthioacetate (SATA reagent/Pierce, Rockford, IL, USA).

For this purpose, to 20 µl of a solution of the IPO1 oligonucleotide in H_2O (concentration: 0.5 µg/ml) were added 2.3 µl of each a sodium carbonate buffer (300 mM, pH 9.0) and a solution of 115 mM of SATA in DMF and incubated for 30 minutes at room temperature with gentle shaking. Subsequently, rebuffering was effected via a HiTrapTM column (1.6 x 2.5 cm, Sephadex G-25 superfine/Pharmacia, Uppsala, Sweden) using an FPLC system (Pharmacia). A 100 mM sodium phosphate buffer, pH 6.5, was used as the elution buffer.

Step 2: Preparation of the maleimide modified antibody

To 90 µl of a 100 mM sodium phosphate buffer, pH 7.0, were added 12.5 µl of a solution of the 6H4 antibody (concentration: 2 mg/ml). This solution was dialyzed in a Slide-A-Lyzer dialysis cassette (10 000 MWCO, sample volume: 0.1-0.5 ml; Pierce, Rockford, IL, USA) overnight at a temperature of +4°C against a 100 mM sodium

phosphate buffer, pH 7.0. Then 15 µl of an aqueous solution of sulphosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (Sulfo-SMCC; Pierce) were added (concentrations: 2.75 mM Sulfo-SMCC, 100 mM sodium phosphate, 1.5% DMF, pH 7.0). After 30 min of incubation at room temperature and with gentle shaking, rebuffering was effected as described in step 1 via a HiTrapTM column (1.6 x 2.5 cm).

Step 3: Preparation and purification of the DNA antibody conjugate

For the preparation of the antibody/DNA conjugate the derivatives prepared in steps 1 and 2 were combined (about 1.5 ml of each the solution of the acetylthioacetyl derivatized DNA and the maleimide modified antibody, respecively).

After starting the coupling reaction by adding 2 µl of an 1M aqueous hydroxylamine hydrochloride solution, pH 7.0, containing 50 mM EDTA, incubation was effected for 2 hours at room temperature in the absence of light and with gentle shaking. Then the reaction was stopped by adding 2 µl of a 10 mM solution of N-ethylmaleimide (Pierce) in DMF. The reaction mixture was concentrated using a Centricon 3 concentrator (Amicon, Beverly, MA, USA) by centrifugation at 7000 x g.

The concentrated reaction mixture (about 500 µl) was subsequently repurified by gel filtration using the Pharmacia FPLC system (Pharmacia, Uppsala, Sweden).

Chromatography conditions:

Column: XK 16 mm/120 mm, filled with Sephacryl 300 HR

(Pharmacia)

elution buffer: 200 mM sodium phosphate buffer, pH 7.0

flow rate: 0.5 ml/min

detection: 280 nm

fraction size: 0.25 ml

The fractions in the elution maximum were collected and used in various dilutions for the immuno PCR.

SEQUENCE LISTING

<110> BAXTER AKTIENGESELLSCHAFT

<120> Method for detecting antigens

<130> Prion Screening

<140>
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CLAIMS:

1. A method for the detection of antigens in a sample by amplifying a nucleic acid bound to a ligand specific for said antigens, characterized in that the detection takes place in the presence of at least one internal standard.

2. A method according to claim 1, characterized in that a nucleic acid bound to a ligand is used as an internal standard.

3. A method according to claim 2, characterized in that the internal standard contains a standard nucleic acid which differs from the nucleic acid used to detect the antigen in at least one detectable feature.

4. A method according to claims 2 or 3, characterized in that the ligand of the standard nucleic acid is different from the ligand binding to the antigen to be detected.

5. A method according to any one of claims 1 to 4, characterized in that said antigens are detected in a sample of a mammalian body fluid.

6. A method according to claim 5, characterized in that said antigens are detected in a sample of a material selected from the group of blood, plasma, a plasma fraction, a blood cell fraction and a preparation of a purified plasmatic protein.

7. A method according to any one of claims 1 to 4,

characterized in that said antigens are detected in a sample of a cell culture.

8. A method according to any one of claims 1 to 7, characterized in that modified proteins are detected as antigens.

9. A method according to any one of claims 1 to 8, characterized in that the modified proteins are obtained by an induced alteration in their conformation.

10. A method according to any one of claims 1 to 9, characterized in that antibodies, particularly monoclonal antibodies, are used as ligands.

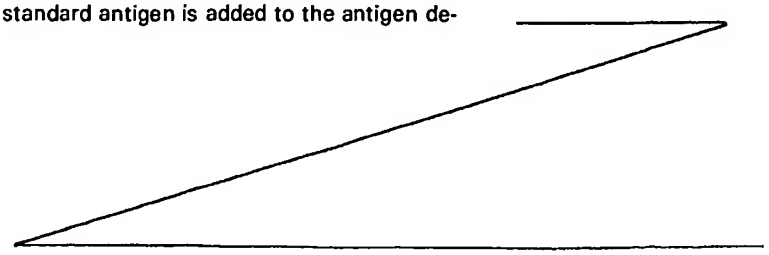
11. A method according to any one of claims 1 to 10, characterized in that said nucleic acid is bound covalently to the ligand.

12. A method according to any one of claims 1 to 10, characterized in that said nucleic acid is bound to said ligand via high affinity ligands.

13. A method according to any one of claims 2 to 12, characterized in that said standard nucleic acid is bound to a ligand which differs from the ligand specific for the antigen to be detected.

14. A method according to any one of claims 1 to 13, characterized in that a standard antigen is detected with the internal standard.

15. A method according to claim 14, characterized in that the standard antigen is added to the antigen de-



tection mixture before or simultaneously with the sample, respectively.

16. A method according to claim 14, characterized in that the standard antigen is already present naturally in the sample.

17. A method according to any one of claims 2 to 16, characterized in that said standard nucleic acid is bound to the ligand specific for the antigen to be detected.

18. A method according to any one of claims 1 to 17, characterized in that said nucleic acid is amplified by a polymerase chain reaction.

19. A method according to any one of claims 2 to 18, characterized in that said standard nucleic acid and the nucleic acid bound to a ligand specific for the antigens to be detected are treated with the same means for amplification, particularly the same primers.

20. A method according to any one of claims 3 to 19, characterized in that said standard nucleic acid differs in length from the nucleic acid bound to a ligand specific for the antigens to be detected.

21. A method according to any one of claims 1 to 20, characterized in that said internal standard is employed at a concentration of slightly above the limit of detection.

22. A method according to any one of claims 1 to 21, characterized in that at least two different standard nucleic acids are empolyed as the internal standard.

23. A reagent to be used in a method according to any one of claims 1 to 22 containing

a) a reactant bound to a ligand specific for the antigen to be detected, and

b) a reactant as the internal standard differing from the reactant of a) in its way of reaction.

24. A reagent according to claim 23, characterized in that the reactants are nucleic acids and that the nucleic acid of b) differs from the nucleic acid of a) in at least one detectable feature.

25. A reagent to be used in a method according to claims 8 or 9 containing a nucleic acid bound to an antibody, which antibody is specific for the modified proteins.

26. A reagent according to claims 24 or 25, characterized in that a standard nucleic acid bound to a ligand is contained as the internal standard, which ligand is further bound to a standard antigen.

27. A reagent according to claim 26, characterized in that the standard antigen is the same as the antigen to be detected.

28. A reagent according to any one of claims 23 to 26, characterized in that a standard nucleic acid bound to a ligand is contained as the internal standard, which

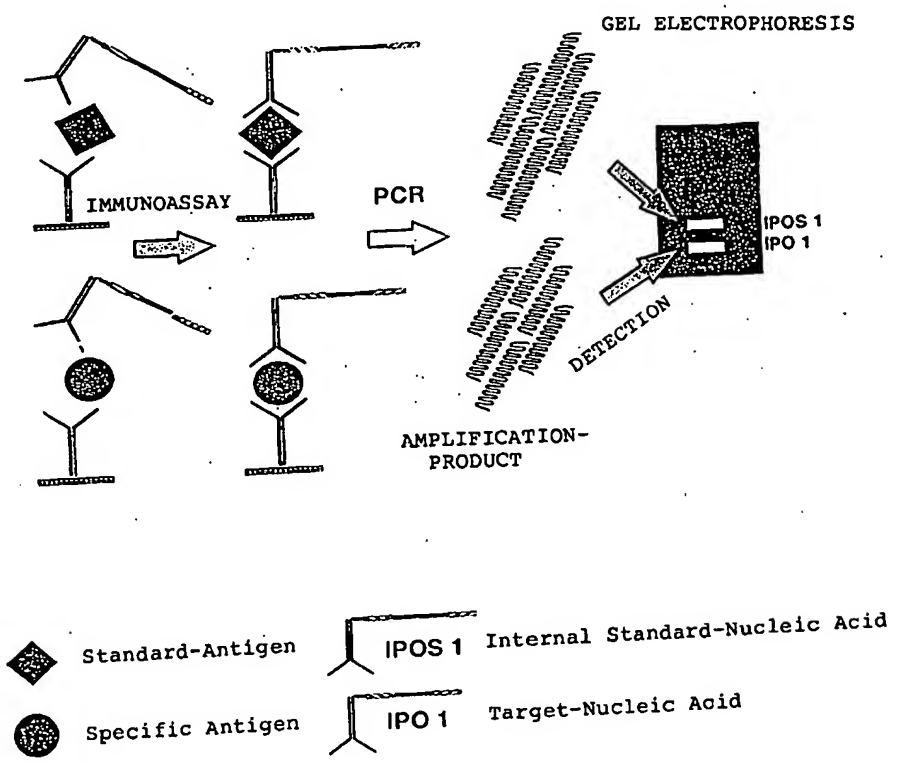
is specific for an antigen other than the antigen to be detected.

29. Use of the method according to any one of claims 1 to 22 for the quantitation of antigens of a pathogen in a mammalian sample.

30. Use of the method according to any one of claims 1 to 22 for the quantitation of antigens of a human pathogen in a biological medicament.

1/1

FIG. 1


GEL ELECTROPHORESIS
IMMUNOASSAY
PCR
IPOS 1
IPO 1
DETECTION
AMPLIFICATION-
PRODUCT
Standard-Antigen
Specific Antigen
IPOS 1
Internal Standard-Nucleic Acid
IPO 1
Target-Nucleic Acid